Resistance to Cadmium Ions and Formation of a Cadmium-Binding Complex in Various Wild-Type Yeasts

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The resistance to cadmium ions (Cd-resistance) and possible formation of cadmium-binding complexes were examined in eight different wild-type yeasts. *Saccharomyces exiguus*, *Pichia farinosa*, *Torulaspora delbrueckii* and *Schizosaccharomyces octosporus* exhibited partial Cd-resistance, as compared to the Cd-resistant strain 301N and the Cu-resistant but Cd-sensitive strain X2180-1B of *Saccharomyces cerevisiae*. *Saccharomyces carlsbergensis*, *Pichia yestis* exiguus, *Pichia farinosa*, *Torulaspora delbrueckii* and *Kluyveromyces lactis* were all Cd-sensitive. The partially Cd-sensitive species, with the exception of *S. exiguus*, accumulated Cd ions in the cytoplasmic fraction to varying extents. This fraction from *S. octosporus* included a Cd-binding complex that contained \((\gamma EC)_n G\) peptides known as cadystins or phytochelatins, while *P. farinosa* and *T. delbrueckii* synthesized Cd-binding proteins that were similar to the Cd-metallothionein produced by *S. cerevisiae* 301N in terms of molecular weight and amino acid composition. These results suggest that such cytoplasmic molecules play a role in the Cd-tolerance of the above three species of yeast. *S. exiguus* retained most cadmium in the cell wall fraction and no Cd-binding complex was found in the cytoplasm, an indication of the important role of the cell wall in its Cd-tolerance. Different modes of binding of Cd ions appear to be involved in the Cd-resistance of wild-type yeasts and fungi.

Key words: Cadmium-binding complex — Cadmium ion stress — Cell wall — Metallothionein — Phytochelatin — Yeast (various species).

Heavy metal ions in small quantities are required for various physiological processes and the normal functions of cells in plants and animals. Elevated levels of such metal ions are generally toxic and cause major damage to cells. In addition to the utilization of metal ions as essential elements, adjustment of intracellular levels of free ions by binding to macromolecules or other mechanisms is indispensable if cells are to protect themselves against excessive metal ions or changes in levels of such ions in the environment (Woolhouse 1983, Webb 1987, Bremner and Beattie 1990, Mehra and Winge 1991).

Two different classes of cytoplasmic molecules that participate in binding of metal ions and, thus, in resistance to metal ions have been identified in various plant and animal cells. Animal cells produce heavy metal-binding proteins known as metallothioneins (MTs; Kagi and Kojima 1987) and plant cells produce \((\gamma EC)_n G\) peptides \((n > 1)\) known as cadystins or phytochelatins (PCs; Murasugi et al. 1981, 1984, Grill et al. 1985). Both types of molecule are rich in cysteine residues as metal-binding sites but they are very different from each other in that the former are synthesized via an mRNA transcript, while the latter are generated from GSH by PC synthase (Scheller et al. 1987, Grill et al. 1989). It is important to determine why such different systems became established and are exploited by plant and animal cells.

Both MT and PC have been identified in several species of yeast and other fungi. This finding implies that fungi might be located at an intermediate position between plants and animals in terms of the binding of heavy metal ions and, thus, in terms of the utilization of such ions. *Saccharomyces cerevisiae*, *Candida glabrata*, *Neurospora crassa*, and *Agaricus bisporus* produce Cu-MT under copper stress (Winge et al. 1985, Mehra et al. 1988, Lerch 1980, Münger and Lerch 1985). By contrast, *Schizosaccharomyces pombe* and *C. glabrata* synthesize a Cd-binding PC complex in response to Cd\(^{2+}\) ions (Kondo et al. 1984, Mehra et al. 1988). *C. glabrata* is unique in that it can produce both MT and PC, depending on the metal ion signal, either Cu\(^{2+}\) or Cd\(^{2+}\) ions (Mehra and Winge 1991). However, the metal-specific regulation of the synthesis of PC and MT in these fungi has not been fully characterized.

We found recently that a Cd-resistant strain (301N) of *S. cerevisiae* was able to produce MT in response to Cd\(^{2+}\) ions, as well as in response to Cu\(^{2+}\) ions (Inouhe et al. 1989, 1991). This finding suggested that yeasts and other fungi have the potential ability to produce MT in response to Cd\(^{2+}\) ions, as do animal cells. Thus, a re-examination of the production of Cd-binding molecules by yeasts, in particular, the many other wild-type species, seems to be quite important for a general understanding of the functions of MT and PC in fungi and other organisms. In the present study, therefore, we examined the effects of Cd\(^{2+}\) ions on cell growth and the possible formation of Cd-binding complex.
plexes in various wild-type yeast.

**Materials and Methods**

**Yeast species and strains**—Two strains (301N and X2180-1B) of *S. cerevisiae* were used as control strains in the present study. The former exhibits high-level resistance to Cd^{2+} ions (Cd-resistance) by producing Cd-MT and the latter is very Cd-sensitive, as are other wild-type strains of *S. cerevisiae* (Inoue et al. 1991). The strain 301N (MATa, ural, CUP1) was taken from our laboratory stocks (Inoue et al. 1989) and the strain X2180-1B (MATa, SUC2, mal, mei, gal2, CUP1) was obtained from the Yeast Genetic Stock Center (University of California, Berkeley, CA, U.S.A.). All wild-type strains of various yeasts were obtained from other laboratories: two strains of *K. lactis* were donated by Drs N. Guge and K. Fukuda of the Kumamoto Institute of Technology, two strains of *S. exiguus* were donated by Dr T. Hisatomi of Fukuyama University, and other yeast strains were donated by Drs Y. Tamai and Y. Watanabe of Ehime University.

**Culture of yeast cells**—Yeast cells were generally grown for 48 h at 30°C in liquid YEPD medium that contained 1% yeast extract (Difco), 2% Bacto-polypeptone (Difco) and 2% glucose. They were stored on 2% agar plates that were prepared with the same medium in test tubes at 4°C. *Z. rouxii* were stored on the same agar medium supplemented with 5% NaCl. Yeast cells picked from the stock cultures in test tubes were transferred to 100 ml of liquid medium that contained 0.5% KH_{2}PO_{4}, 0.25% MgSO_{4}·7H_{2}O, 0.5% polypeptone, 0.4% yeast extract (Difco) and 2% glucose and grown for 48–72 h. Precultured cells were inoculated in the same liquid medium that was supplemented with 0.03–0.3 mM CdSO_{4} and grown for 48 h. The cells were collected by centrifugation and dry weights were determined as a measure of cell growth. Alternatively, yeast cells that had been precultured in the absence of Cd^{2+} ions were streaked on an agar medium plate with a linear gradient of concentrations of Cd^{2+} ions (0–1 mM) and grown for 72–96 h at 30°C. The length of the band on which visible yeast cells grew up was taken as an index of Cd-tolerance of cells.

**Analysis by gel-permeation chromatography of Cd-binding proteins**—Yeast cells (2 g FW), grown in an appropriate liquid medium, were collected by centrifugation at 1,500 × g and the cells were homogenized in 2 ml of 20 mM Tris-HCl (pH 7.9) in a homogenizer (Braun, Melsungen, Germany) with glass beads. The homogenate was turned on five times for 2 min each and cells were homogenized at 0°C under N_{2} gas. Each homogenate was heated at 70°C for 3 min and centrifuged at 100,000 × g for 60 min to remove heat-labile proteins. The supernatant was subjected to GPC on a column (15 mm i.d. × 1,200 mm) of Sephadex G-50 (Pharmacia, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl (pH 7.9) that contained 0.2 M NaCl. The column was eluted with the same solution at 0.75 ml min⁻¹. The elution pattern was monitored by UV absorption at 250 and 280 nm to estimate the distributions of Cd-SH complex and protein, respectively. The Cd-binding MT from *S. cerevisiae* 301N was used as a standard MT (molecular weight: 9,000). The fractions that contained Cd-binding complexes that corresponded to Cd-MT were collected and stored at −30°C.

**Analysis of the amino acid compositions of Cd-binding complexes from the yeasts**—The amino acid compositions of the Cd-binding complexes from the yeasts were determined with an amino acid analyzer (K-202SN; Kyowa Seimitsu, Tokyo, Japan) after hydrolysis in 6 M HCl at 110°C for 12 h under N_{2}. The cysteine content was determined as cysteic acid after performic acid oxidation (Hirs 1967) and is expressed in term of half-cystine moieties.

**Analysis of Cd-binding peptides by HPLC**—Yeast cells (0.1 mg FW) were extracted with 0.1 ml of sulfo salicylic acid for 1 h at 0°C and the extract was centrifuged at 1,500 × g to remove cell debris. The supernatant was collected and reacted with PCMB. Then the reaction mixture was subjected to HPLC as described previously (Inoue et al. 1994). In brief, a reverse-phase column was used (Hibar Lichrosorb RP-18; Cica-Merk, Darmstadt, Germany), with elution with a linear gradient of 20% to 40% acetonitrile in 0.1%TFA for 40 min. Peaks of (yEC)_{2}P peptides (n = 2 to 5) were quantified by monitoring absorbance at 254 nm (A_{254}) with corresponding authentic peptides as standards (Matsunoto et al. 1990).

**Determination of levels of protein and cadmium**—Proteins were quantitated by the method of Lowry et al. (1951) or by monitoring absorbance at 280 nm. Amounts of cadmium in metal-binding proteins were determined with an atomic absorption spectrophotometer (207; Hitachi, Tokyo, Japan) after wet combustion. The cadmium content of yeast cells was determined after hydrolysis with a mixture of HNO_{3} and HClO (10 : 1, v/v) for 30 min, as reported previously (Inoue et al. 1994).

**Results**

**Effect of Cd^{2+} ions on the growth of yeast cells**—The effects of Cd^{2+} ions on the growth of various yeasts were examined both in liquid medium and on agar-solidified medium (Fig. 1). As shown in Figure 1A, the Cd-resistant strain 301N of *S. cerevisiae* exhibited almost complete resistance to 0.3 mM Cd^{2+} ions, while the Cu-resistant strain X2180-1B was sensitive to 0.03 mM Cd^{2+} ions in liquid medium. The other wild-type yeast cells were unable to grow in the presence of 0.3 mM Cd^{2+} ions. However, *S. exiguus*, *P. farinosa*, *T. delbrueckii* and *S. octosporus* exhibited partial resistance to Cd^{2+} ions at 0.03–0.1 mM. All these yeast also exhibited substantial resistance to Cd^{2+} ions on agar medium plates with a gradient of Cd^{2+} ions (Fig. 1B).

**The uptake and distribution of cadmium in yeast cells**—Figure 2 shows details of the uptake of Cd^{2+} ions by yeast cells that exhibited partial resistance to 0.1 mM Cd^{2+} ions and the distribution of Cd^{2+} ions in the soluble fractions of cells. Cells of *S. exiguus*, *T. delbrueckii* and *S. octosporus* retained larger amounts of Cd^{2+} ions than did those of *S. cerevisiae* 301N (Fig. 2A). *P. farinosa* took up Cd^{2+} ions but to a lesser extent than did 301N. Approximately 37% of the Cd^{2+} ions taken up by cells were localized in the soluble fraction of 301N. This value was 18% for *S. octosporus*, 4% for *T. delbrueckii*, and 2.5% for *P. farinosa* (Fig. 2B). However, few or no Cd^{2+} ions were detected in the soluble fractions of two strains of *S. exiguus*, THE1-16B or THE1-16C. These results suggest that *S. exiguus* has a different mode of Cd-binding and, thus, of Cd-resistance from the other yeast species.

**Cd-binding complexes in yeast cells**—Cytoplasmic fractions prepared from Cd-resistant yeast cells were fractionated on a column of Sephadex G-50 in an attempt to de-
Resistance to and binding of Cd in various yeasts

S. cerevisiae 301N
S. cerevisiae X2180-1B
S. carlsbergensis
S. exigua THE1-16B
S. exigua THE1-16C
K. lactis NK40
K. lactis KA5-6C
P. farinosa
P. mogii
Z. rouxii
T. delbrueckii
S. octosporus

Fig. 1 Effects of Cd$^{2+}$ ions on the growth of various yeast cells. Yeast cells were grown for 48 h at 30°C in a liquid medium that contained various concentrations of CdSO$_4$ (A). Alternatively, yeast cells that had been precultured for 48 h in liquid medium were streaked on an agar plate that had been prepared with medium with a gradient of Cd$^{2+}$ ions from 0 to 1 mM (B). For details, see text.

tect Cd-binding complexes (Fig. 3). P. farinosa, T. delbrueckii and S. octosporus each produced a Cd-binding complex that was collected from fractions 20 to 40. The location of the peak of the Cd-binding complex of the first two yeasts was identical to that of Cd-MT of 301N (data not shown), suggesting the production of MT-like proteins with similar molecular weights. The molecular weight of the Cd-binding complex from S. octosporus appeared to slightly larger than that of Cd-MT. S. exigua THE1-16B did not produce a Cd-binding complex and no other Cd-containing peaks were obtained from the cytoplasmic fraction. When cytoplasmic fractions of S. carlsbergensis, P. mogii, Z. rouxii, and K. lactis that had been grown in the presence of 0.03 mM Cd$^{2+}$ ions were analyzed under the same conditions, most Cd$^{2+}$ ions were eluted in low-molecular-weight fractions (tubes 50–60) and Cd$^{2+}$ ions were not

Fig. 2 Uptake of Cd$^{2+}$ ions by yeast cells (A) and the distribution of Cd$^{2+}$ ions in the soluble fractions of the cells (% to total Cd; B). Yeast cells that exhibited Cd-resistance were grown for 48 h in the presence of 0.1 mM Cd$^{2+}$ ions. The Cd content of cells was determined after hydrolysis with a mixture of HNO$_3$ and HClO. The soluble fraction was extracted by homogenization and then subjected to determination of the Cd content. For details, see text.

Fig. 3 Gel-permeation chromatography of Cd-binding complexes in soluble fractions of several yeasts. Yeast cells that exhibited partial Cd-resistance were grown for 48 h in the presence of Cd$^{2+}$ ions. The cells were extracted with 20 mM Tris-HCl (pH 7.9) and the extracts were applied to a column of Sephadex G-50. Absorbance at 250 and 280 nm and the Cd content of each fraction (3 ml) were measured. A, P. farinosa; B, T. delbrueckii; C, S. octosporus; D, S. exigua THE1-16B.
Table 1 Amino acid compositions of yeast Cd-binding complexes

<table>
<thead>
<tr>
<th>Species</th>
<th>P. farinosa 30-38</th>
<th>T. delbrueckii 30-38</th>
<th>S. octosporus 28-38</th>
</tr>
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<tbody>
<tr>
<td>Asp</td>
<td>11.6</td>
<td>12.9</td>
<td>4.2</td>
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<tr>
<td>Thr</td>
<td>4.5</td>
<td>3.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Ser</td>
<td>10.2</td>
<td>7.3</td>
<td>3.5</td>
</tr>
<tr>
<td>Glu</td>
<td>14.6</td>
<td>13.4</td>
<td>27.9</td>
</tr>
<tr>
<td>Pro</td>
<td>1.9</td>
<td>1.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Gly</td>
<td>14.7</td>
<td>14.4</td>
<td>9.6</td>
</tr>
<tr>
<td>Ala</td>
<td>2.7</td>
<td>2.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Cys/2</td>
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<td>17.9</td>
<td>25.9</td>
</tr>
<tr>
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<td>1.8</td>
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<tr>
<td>Met</td>
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</tr>
<tr>
<td>Ile</td>
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<tr>
<td>Leu</td>
<td>5.7</td>
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<tr>
<td>Tyr</td>
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<tr>
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<td>7.3</td>
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<td>7.6</td>
</tr>
<tr>
<td>Arg</td>
<td>3.2</td>
<td>1.9</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Partially purified Cd-binding complexes were hydrolyzed by treatment with 6 M HCl for 12 h at 110°C and released amino acids were quantitated with an amino acid analyzer. Cysteine was determined as the half-cystine content after performic acid oxidation. Data are expressed as mol% of total amino acids.

"Fractions refer to tube numbers in Fig. 3.

Amino acid compositions of Cd-binding complexes—The major fractions containing Cd-binding complexes after GPC were collected and the amino acid compositions of the various complexes were determined (Table 1). The Cd-binding complex from S. octosporus was mainly composed of Glu, Cys and Gly residues, at a molar ratio of ca. 3:3:1. Complexes from T. delbrueckii and P. farinosa were rich in other amino acid residues but lacked aromatic amino acids. The amino acid compositions of the latter two species exhibited some similarity (correlation coefficients, r = 0.92 and 0.89, respectively) to that of Cd-MT produced by S. cerevisiae 301N (Inouhe et al. 1991).

PC peptides in various yeast cells—To examine the possible formation of PC peptides in yeast cells exposed to Cd²⁺ ions, the acid extracts of yeast cells were analyzed by HPLC (Fig. 4). A large peak of (yEC)₃G was detected in the case of extracts of S. octosporus, with a retention time of 23.6 min, but such a peak was not found in the analysis of other extracts (data not shown). S. octosporus also synthesized other (yEC)ₙG peptides with n = 2, 4, 5 and 6, at relative levels of 31.5, 2.3, 0.3 and 0.2%, respectively. These compounds were also totally absent from the other yeast cells (data not shown). These results suggest that S. octosporus is capable of producing PC peptides in response to Cd²⁺ ions and that the other yeasts might not be. Small but clear peaks near the retention time of authentic (yEC)₃G were detected occasionally in the case of S. exigus THE1-16B, P. farinosa, and T. delbrueckii, as well as in the case of Cd-sensitive K. lactis KAS-6C, S. cerevisiae X2180-1B and S. carlsbergensis (data not shown).

Discussion

In the present study, we demonstrated that various yeasts with no obvious prior exposure to Cd²⁺ ions exhibited different growth responses to Cd²⁺ ions with respect to each other and to known Cd-resistant or sensitive strains of S. cerevisiae. These results indicate that the responses to Cd²⁺ ions are not as simple as the responses of some laboratory-generated mutants that were selected under the influence of specific metal ions. It is generally accepted that Cd²⁺ ions are not essential for the growth of plants and microorganisms, unlike the other heavy metal ions, such as Cu²⁺, Zn²⁺, Mo²⁺ and Mn²⁺. Thus, the Cd-resistance of wild-type yeasts can be explained as a by-product but not as a direct product of a "natural resistance mechanism", which can be defined as being specific for essential metal ions and might, thus, involve possible alterations in pre-existing metabolism or the regulation of uptake specific to essential ions. It is not easy to validate but it may
be very important to research the historical backgrounds and ecotypes of various yeasts, if we are to understand their different phenotypes with respect to Cd-resistance. However, first of all, an analysis of the biochemical backgrounds of the Cd-resistance mechanisms expressed in the respective species is required.

Among the wild-type yeasts that we examined, four species had greater Cd-resistance than the others, but they were less resistant than the 301N strain of *S. cerevisiae*. Most of the wild-type yeasts took up greater amounts of Cd\(^{2+}\) ions into cells than did the 301N strain (Fig. 2 and unpublished data). This observation indicates that these yeasts have no special way of reducing the uptake of Cd\(^{2+}\) ions to prevent the accumulation of these ions in the cells. Therefore, their substantial Cd-resistance might be due to some intracellular mode of detoxification or bindings of Cd\(^{2+}\) ions in a subcellular fraction. The exception was *P. farinosa*, which took up only small amounts of Cd\(^{2+}\) ions as compared with the other yeasts (Fig. 2). The mechanism and possible function of Cd-resistance in this yeast require further study.

As important Cd-binding complexes that function in the sequestration of cadmium, Cd-MTs have been identified in cytoplasmic fractions of various animal cells. However, little is known about the natural occurrence of Cd-MTs in plants or fungi apart from *S. cerevisiae* 301N. This strain arose by spontaneous mutation of a wild-type strain during continuous exposure to Cd\(^{2+}\) ions (Tohoyama and Murayama 1977) and, thus, it cannot be regarded as a wild-type strain. The present study revealed that two wild-type yeasts, *T. delbrueckii* and *P. farinosa*, were able to produce MT-like Cd-binding proteins in response to Cd\(^{2+}\) ions at a substantial level. These findings support the idea that MTs have some general role in Cd-resistance in fungi as they do in animals. The novel MT-like Cd-binding proteins have some characteristics similar to those of the Cd-MT of 301N. However, their structures are as yet unknown. There remains the possibility that various types of MT will be discovered in those yeast species and other fungi.

*T. delbrueckii* and *P. farinosa* exhibited partial Cd-resistance and produced MT-like proteins. *K. lactis* and some other Cd-sensitive yeasts were unable to produce Cd-MT. Macreadie et al. (1991) reported that the constitutive expression of an incorporated gene for MT in *K. lactis* increased the resistance of cells to Cd\(^{2+}\) ions as well as to Cu\(^{2+}\) ions. These results suggest that MT contributes to the Cd-resistance of yeasts in general, if it is produced. The presence or absence of genes for MT appears to be the important factor that determines formation of Cd-MT and, thus, the Cd-resistance of wild-type yeasts.

The synthesis of MT in *S. cerevisiae* is strictly controlled by Cu\(^{2+}\) (or Ag\(^{+}\)) ions at the transcriptional level of the genes (*CUP1*), and their effect is mediated by a DNA-binding factor called the ACE1 protein (Thiele 1988, Fürst et al. 1989). However, Cd\(^{2+}\) ions cannot enhance the function of ACE1 for expression of the *CUP1* gene. Thus, Cd\(^{2+}\) ions do not induce the synthesis of MT in the yeast. Recently, synthesis of Cd-MT in 301N was shown to be due, in a part, to a mutated heat-shock transcriptional factor (HSF1; Sewell et al. 1995), which exhibits an elevated basal rate of transcription of the *CUP1* gene (Tohoyama et al. 1992). So far, no Cd-specific transcriptional factors have been identified. More detailed analysis of such Cd-specific or nonspecific factors should give us clues to the mechanisms of expression of genes for MT in *T. delbrueckii* and *P. farinosa* and for its absence in other Cd-sensitive yeasts.

In the present study, we found that *S. octosporus* does not synthesize MT but produces high levels of PC peptides. Thus, these peptides appear to have a central role in its Cd-resistance. A member of the same genus, *S. pombe* also produces PCs (Murasugi et al. 1981). The major PCs from *S. octosporus* were (γEC)\(_2\)G and (γEC)\(_3\)G, which are also the predominant PCs produced by *S. pombe* (Kondo et al. 1984). Thus, *Schizosaccharomyces* might be a genus of fungi that can produce PCs as the main Cd-binding complexes under Cd\(^{2+}\) ion stress. PCs are produced by *C. glabrata* (Mehra and Winge 1991) and by many species of higher plants (Grill et al. 1985, Robinson and Jackson 1986, Rauser 1990). Relationships between the production of homologous PCs and the evolutionary positions of plants and fungi are unknown.

The biochemical background of Cd-resistance in *S. exigus* appears to be quite different from that in other wild-type yeasts. This yeast can resist the effects of Cd\(^{2+}\) ions without the involvement of cytoplasmic Cd-binding complexes. It accumulates Cd\(^{2+}\) ions only in a cell-wall fraction. The structure and metabolism of the cell wall have been studied in detail in *S. cerevisiae* and other species, but little is known in the case of *S. exigus*. Further studies on the specificity of the structure and metabolism of the cell wall will help us to understand the unique properties of *S. exigus* with respect to Cd-resistance.

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**References**


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